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The vitamin D_3 analogue, calcipotriol, induces sphingomyelin hydrolysis in human keratinocytes

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Abstract **The possible role of sphingomyelin cycle for the regulation of cell proliferation was investigated in human keratinocytes. The time-dependent breakdown of sphingomyelin was observed in the immortalized human keratinocyte cell line HaCaT as well as in primary human keratinocytes thereby providing evidence that the sphingomyelin cycle might be of importance in the epidermis. Peak levels of 20–30% sphingomyelin hydrolysis were measured 3 h after treatment of the cells with 1α,25-dihydroxyvitamin D_3 or with the vitamin D_3 analogue, calcipotriol. The decrease of sphingomyelin upon addition of vitamin D_3 or calcipotriol was accompanied by an approximately 70% increase of ceramide in the cells. The effects of vitamin D_3 and calcipotriol on sphingomyelin breakdown were paralleled by their antiproliferative potency. Furthermore, the cell-permeable ceramide, *N*-acetylsphingosine, and natural ceramide inhibited cell proliferation of human keratinocytes. The results presented suggest that induction of the sphingomyelin cycle represents one mechanism mediating the therapeutic effect of calcipotriol in treatment of psoriasis.**

Key words: Calcipotriol; Sphingomyelin hydrolysis; Ceramide; 1α,25-Dihydroxyvitamin D_3; Human keratinocyte; Signal transduction; Cell proliferation; Psoriasis

1. Introduction

The sphingomyelin (SM) cycle has been initially identified in HL-60 human leukemia cells as a new cellular signalling pathway in response to vitamin D_3 [1]. It has been shown that a time-dependent and reversible hydrolysis of SM occurs via activation of a sphingomyelinase generating ceramide [2]. Subsequently, the SM cycle is completed by the transfer of a choline head group from phosphatidylcholine to ceramide [3]. Up to now, several other agonists of the SM cycle have been described, including tumor necrosis factor α (TNFα), γ-interferon, dexamethasone, interleukin-1, nerve growth factor (NGF), complement and brefeldin A (for review, see [4,5]). It has been implicated that the SM cycle mediates the effects of these agonists finally leading to terminal differentiation or apoptosis [6,7]. Using cell-permeable ceramide analogues, it was possible to mimick the effects of several inducers of SM hydrolysis on cell proliferation and differentiation [8].

Psoriasis is a disease of the skin which is characterized by an enormous hyperproliferation of keratinocytes and the topical treatment with antiproliferative agents represents a classical concept of therapy [9]. Calcipotriol (MC 903) is a vitamin D_3 analogue which has been found to potently induce cell differentiation and inhibit cell proliferation cells possessing the receptor for 1α,25-dihydroxyvitamin D_3 by an unknown mechanism [10,11]. Furthermore, topical treatment of psoriasis with calcipotriol has been shown to constitute an effective and potent therapy for this skin disease [12]. Therefore, the elucidation of the mechanism underlying the therapeutic effect of calcipotriol is of great interest and might lead to the development of new drugs.

In the present study, the existence of a 1α,25-dihydroxyvitamin D_3 inducible SM cycle was demonstrated in human keratinocytes. Additionally, the vitamin D_3 analogue, calcipotriol, was found to induce the hydrolysis of 20–30% of total cellular SM and to increase the formation of ceramide in a time-dependent and reversible manner, with maximal activity after 3 h. 1α,25-dihydroxyvitamin D_3 as well as calcipotriol inhibited the proliferation of keratinocytes and HaCaT cells and their antiproliferative effects were mimicked by *N*-acetylsphingosine (C_2-ceramide) and natural ceramide. Hence, the SM cycle is discussed as a possible mechanism underlying the antiproliferative effect of calcipotriol in psoriasis.

2. Materials and methods

2.1. Materials

Streptomyces sp. sphingomyelase, dodecane and ceramide (from bovine brain SM or cerebroside) were purchased from Sigma (München, Germany). Solvents and reagents (reagent grade) were obtained from Merck (Darmstadt, Germany) and Fluka (Neu-Ulm, Germany). Triton X-100 was purchased from Aldrich (Steinheim, Germany). [*methyl*-^{3}H]Choline chloride (2.8–3.1 TBq/mmol), [*N-methyl*-^{14}C]SM (2.07 GBq/mmol), L-3-phosphatidyl-[*N-methyl*-^{3}H]choline, 1,2-dipalmitoyl (2.44 TBq/mmol) and L-[3-^{3}H]serine (1.11 TBq/mmol) were from Amersham (Braunschweig, Germany). *N*-Acetylsphingosine (C_2-ceramide) was from Alexis (Grünberg, Germany). 1α,25-dihydroxyvitamin D_3 and calcipotriol (MC 903) were a gift from Dr. L. Binderup (Leo Pharmaceutical Products; Kopenhagen, Denmark). For quantification of radioactivity a Berthold (Wildbad, Germany) LB 2821 HR thin-layer chromatography scanner was used.

2.2. Cell culture

HaCaT cells [13] were grown in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 0.35 g/l glutamine, 100 000 IU/l penicillin and 0.1 g/l streptomycin in plastic culture dishes (Nunc; Wiesbaden, Germany). Media and culture reagents were obtained from Gibco (Karlsruhe, Germany). Penicillin and streptomycin were from Boehringer (Mannheim, Germany). Human keratinocytes were pre-

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Abbreviations: C_2-ceramide, *N*-acetylsphingosine; cpm, counts per minute; HaCaT, immortalized human keratinocyte cell line; HL-60, leukemia cell line; KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; MCDB, cell culture media designed for serum-free growth of specific cell types; MUH, 4-methylumbelliferyl heptanoate; NGF, nerve growth factor; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute; SM, sphingomyelin; TNFα, tumor necrosis factor α.

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pared from human forskin as described recently [14] and cultured in keratinocyte growth medium (KGM). KGM was prepared from keratinocyte basal medium (KBM) by addition of 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.5 μM hydrocortisone, 50 μg/ml bovine pituitary extract, 100 μg/ml penicillin/streptomycin, and 2.5 μg/ml fungizone. KBM consisted of MCDB 153 medium as described [15] which was supplemented with 100 μM ethanolamine, 100 μM phosphoethanolamine, 240 μM histidine, 750 μM isoleucine, 90 μM methionine, 90 μM phenylalanine, 45 μM tryptophan, 75 μM tyrosine and 150 μM $CaCl_2$. KBM and supplements were purchased from Clonetics (San Diego, CA, USA). 1α,25-dihydroxyvitamin D_3 and calcipotriol were diluted into KBM or KGM as described above from 10 μM stock ethanol solutions. Ceramide was first dissolved in ethanol/dodecane (98:2, v/v) at a concentration of 177 μM as described [16]. The solution was then added to KGM and sonicated for 30 min. C_2-ceramide was diluted into KGM from a 30 mM stock dimethyl sulphoxide solution. Ethanol, ethanol/dodecane (98:2, v/v) and dimethyl sulphoxide (vehicles) were added to controls and were present at 1%, 0.56% and 0.1%, respectively. 0.1% dimethyl sulphoxide did not significantly reduce the proliferation rate of human keratinocytes (98% of controls). The same was true for 1% ethanol and 0.56% ethanol/dodecane (98:2, v/v).

2.3. Choline labelling of cells

For choline labelling of HaCaT cells and human keratinocytes, medium was removed and pulse medium (KGM containing 3.7×10^4 Bq/ml [*methyl*-^{3}H]choline) was added. After incubation for 72 h, cells were washed twice with phosphate-buffered saline (PBS) and then treated with 100 nM 1α,25-dihydroxyvitamin D_3 or 100 nM calcipotriol in KBM for different time periods. Cells were harvested in 400 μl ice-cold PBS by use of a cell lifter (Costar; Cambridge, MA, USA). After freeze-drying of the cells, lipids were extracted by a modified method of Bligh and Dyer [17] as described [18]. Total lipid extracts were dried under a stream of nitrogen and stored at −20°C.

2.4. Sphingomyelin quantitation

SM was quantified using bacterial sphingomyelinase to release [^{3}H]phosphocholine as described recently [19]. Briefly, the total lipid extracts were resuspended in 100 μl of assay buffer (100 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 0.1% Triton X-100). The samples were sonicated for 5 min and 1 unit/ml sphingomyelinase from *Streptomyces sp.* was added. After incubation at 37°C for 2 h, the reaction was stopped by addition of 1 ml chloroform/methanol (2:1, v/v) and the liberated [^{3}H]phosphocholine was recovered with the aqueous phase of a Folch extraction [20]. Phase separation was completed by addition of 100 μl water and the aqueous phase was taken for scintillation counting. The radioactivity of the aqueous phase normally reached 10,000 cpm and the radioactivity in control samples was set as 100%. Subsequently, SM in the samples of calcipotriol or 1α,25-dihydroxyvitamin D_3-treated cells was calculated as % of control. The assay conditions described above were verified using [*methyl*-^{14}C]SM and [*methyl*-^{3}H]phosphatidylcholine. As shown in Table 1, maximal SM hydrolysis (> 98%) without accompanying phosphatidylcholine hydrolysis (< 5%) was observed.

Furthermore, SM was also determined by a thin-layer chromatography method. Lipids were spotted on silica gel 60 high-performance thin-layer chromatography plates and plates were developed in chloroform/methanol/triethyl-amine/water (30:35:34:8, by vol). Radioactivity was quantified by radioscanning. SM and phosphatidylcholine were identified by calibrating the scanner with know standards. A comparison of the thin-layer chromatography method with the sphingomyelinase method demonstrated that the methods gave identical results (23% SM-hydrolysis as compared with control after 60 min of stimulation with TNFα).

2.5. Determination of cellular ceramide

The formation of cellular ceramide upon addition of 1α,25-dihydroxyvitamin D_3 or calcipotriol was determined as described [21]. Briefly, subconfluent cells were radiolabelled by the addition of L-[^{3}H]serine (2 μCi/ml) to serine-free Minimal Essential Medium (Gibco; Karlsruhe, Germany) containing 5% heat-inactivated fetal calf serum for 24 h. The confluent cells were washed twice with PBS and incubated for 5 h in KGM containing unlabelled serine (5 mM). The cells were washed as before and then treated with 100 nM 1α,25-dihydroxyvitamin D_3 or 100 nM calcipotriol in KBM for different time periods. The medium was removed, and the cells were precipitated in 0.5 ml methanol. Lipids were extracted by addition of 0.25 ml chloroform and incubation for 1 h followed by addition of 0.75 ml chloroform and incubation for a further 30 min. The phases were separated by the addition of 0.75 ml water and the organic phase was removed and dried under a stream of nitrogen. Lipids were solubilized in chloroform/methanol (2:1, v/v), separated by high-performance thin-layer chromatography using the solvent system chloroform/methanol (9:1, v/v), and radioactivity was quantified by radioscanning. Ceramide was identified by calibrating the scanner with radiolabelled ceramide which was prepared by incubation of serine-labelled cells with 0.1 unit/ml sphingomyelinase. The ceramide-bound radioactivity of control samples at time point 0 was 0.66% of total radioactivity. Total radioactivity in the samples normally reached 50,000 cpm. The ceramide-bound radioactivity of control samples was set as 100% and ceramide in the samples of 1α,25-dihydroxyvitamin D_3 or calcipotriol-treated cells was calculated as % of control.

2.6. Cell proliferation assays

Human keratinocytes and HaCaT cells were seeded in 96-multiwell plastic culture dishes with a density of 5×10^3 cells/cm^2 and treated with different concentrations of 1α,25-dihydroxyvitamin D_3, calcipotriol, natural ceramide or C_2-ceramide in KGM. After incubation for 24–72 h, the cells were washed twice with PBS and cell proliferation was determined by the MUH fluorescence assay using 4-methylumbelliferyl heptanoate (Serva, Heidelberg, Germany) as described recently [22]. The absolute fluorescence units of control cells were set as 100% and the proliferation rate of treated cells was calculated as % of control. Additionally, cell proliferation was determined by the crystal violet assay as described in detail [23]. Both assays gave similar results.

2.7. Other procedures

Statistical comparisons were made in these studies with Student's *t*-test.

3. Results and discussion

3.1. Calcipotriol time-dependently activates sphingomyelin hydrolysis

In keratinocytes, the existence of the SM cycle has not been

Table 1
Distribution of radioactivity between the aqueous and organic phases after sphingomyelinase treatment

Sample	Radioactivity in the aqueous phase (% of total cpm)	Radioactivity in the organic phase (% of total cpm)
[*methyl*-^{14}C]SM + enzyme	98.4	1.6
[*methyl*-^{14}C]SM − enzyme	0.7	99.3
[*methyl*-^{3}H]phosphatidylcholine + enzyme	4.2	95.8
[*methyl*-^{3}H]phosphatidylcholine − enzyme	1.3	98.7

Trace-amounts of [*methyl*-^{14}C]SM (44,000 dpm) or [*methyl*-^{3}H]phosphatidylcholine (440,000 dpm) were added to a total lipid extract from HaCaT cells. Then, sphingomyelinase assays were performed as described in section 2 and 400 μl of the aqueous phases and 800 μl of the organic phases were taken for scintillation counting. Values are given as % of total radioactivity and represent the mean of two determinations with an error less than 1%. The experiment was repeated with purified SM and purified phosphatidylcholine and similar results were obtained.

demonstrated yet. We therefore tested the known inducer of SM hydrolysis, 1α,25-dihydroxyvitamin D_3, in human keratinocytes and in the immortalized human keratinocyte cell line HaCaT. As expected, a time-dependent effect of 1α,25-dihydroxyvitamin D_3 on SM hydrolysis occurred in HaCaT cells (Fig. 1A). The hydrolysis of approximately 25% of total cellular SM was observed after 3 h of 1α,25-dihydroxyvitamin D_3 treatment and SM levels returned to control levels by 6 h. Similar results were obtained in primary keratinocytes (data not shown). The amount of hydrolyzed SM and the time course of SM hydrolysis are in accordance with results obtained in the leukemic cell line HL-60 [1]. TNFα, another well known inducer of the SM cycle [19,24], also activated SM hydrolysis in keratinocytes and HaCaT cells (data not shown). Thus, our results provide strong experimental evidence that the agonist-stimulated hydrolysis of SM represents an operative signal transduction pathway in keratinocytes. This might be of future interest since both SM and sphingomyelinase are present in the epidermis [25,26] and the importance of ceramides in the skin barrier system has been discussed [27].

In another set of experiments, we investigated the effects of the vitamin D_3 analogue, calcipotriol, on SM hydrolysis. As shown in Fig. 1B, incubation of HaCaT cells with 100 nM calcipotriol led to the time-dependent hydrolysis of SM. Maximal effects of approximately 30% SM hydrolysis were observed 3 h after treatment with calcipotriol and the time course of the calcipotriol effect resembled the vitamin D_3 effect described in the previous paragraph. Furthermore, nearly the same effects of calcipotriol were observed in primary keratinocytes (data not shown). The known activators of the SM cycle may be categorized by the temporal nature of their activation. Early activators, such as TNFα and interleukin-1, cause SM hydrolysis within 15–30 min whereas late activators, such as vitamin D_3 and γ-interferon, show stimulation of SM hydrolysis after 2 h of treatment [3]. From the results obtained we suggest that calcipotriol is a late inducer of the SM cycle.

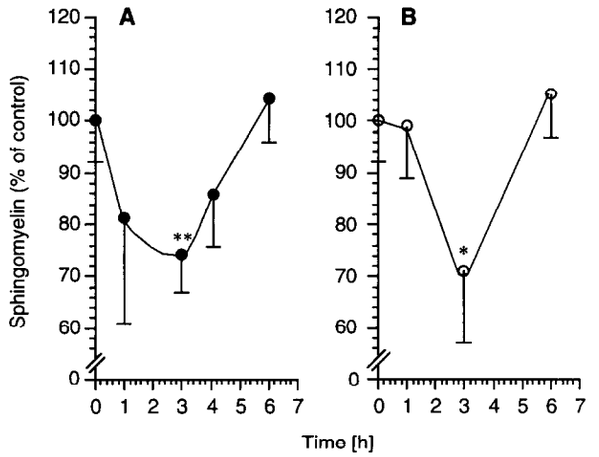

Fig. 1. Time-dependent hydrolysis of sphingomyelin after 1α,25-dihydroxyvitamin D_3 or calcipotriol treatment of HaCaT cells. Subconfluent HaCaT cells were labelled for 72 h with pulse medium containing 3.7×10^4 Bq/ml [*methyl*-^{3}H]choline. Pulse medium was removed and the cells were treated with 100 nM 1α,25-dihydroxyvitamin D_3 (A) or calcipotriol (B). Control media contained 1% ethanol. After different incubation times as indicated in the figure, the cells were harvested and SM was quantitated as described in section 2. Values represent the mean of three experiments and the standard deviation is shown as a vertical bar. *Significantly different from controls at $P < 0.05$. **Significantly different from controls at $P < 0.02$.

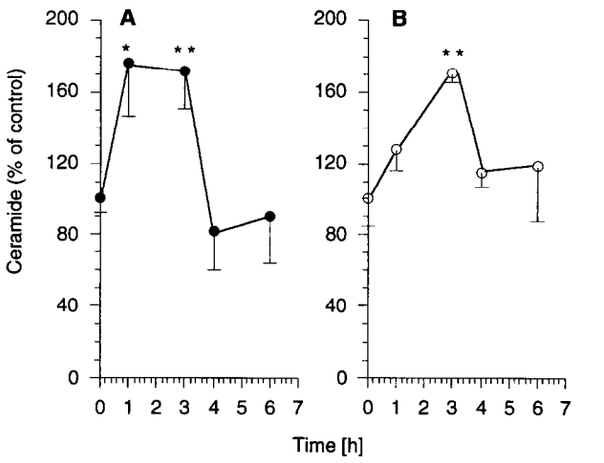

Fig. 2. Time-dependent formation of ceramide after 1α,25-dihydroxyvitamin D_3 or calcipotriol treatment of HaCaT cells. Subconfluent HaCaT cells were labelled for 24 h with serine-free medium containing 7.4×10^4 Bq/ml L-[^{3}H]serine. Pulse medium was removed and the cells were incubated for 5 h with KGM containing 5 mM unlabelled serine. Then, the cells were treated with 100 nM 1α,25-dihydroxyvitamin D_3 (A) or calcipotriol (B). Control media contained 1% ethanol. After different incubation times as indicated in the figure, the cells were harvested and cellular ceramide was quantitated as described in section 2. Values represent the mean of three experiments and the standard deviation is shown as a vertical bar. *Significantly different from controls at $P < 0.02$. **Significantly different from controls at $P < 0.01$.

3.2. *Calcipotriol time-dependently induces formation of ceramide*

It has been shown that treatment of HL-60 cells with vitamin D_3 resulted in an increase of cellular ceramide with peak levels after 2 h [1]. To demonstrate that vitamin D_3 as well as calcipotriol not only activated SM hydrolysis but at the same time induced the formation of cellular ceramide we metabolically labelled the lipid backbone of SM by addition of L-[^{3}H]serine to the medium of the cells. When keratinocytes (data not shown) or HaCaT cells were treated with either vitamin D_3 (Fig. 2A) or calcipotriol (Fig. 2B) an approximately 70% increase of cellular ceramide was observed after 3 h and ceramide returned to base-line levels by 4–6 h. The time-dependent formation of ceramide is in accordance with the time-course of SM hydrolysis thereby confirming the metabolic pathway involved in SM hydrolysis which has been characterized in HL-60 cells [1].

3.3. *Antiproliferative effects of calcipotriol, 1α,25-dihydroxyvitamin D_3, natural ceramide and C_2-ceramide on human keratinocytes and HaCaT cells*

To investigate the antiproliferative effect of calcipotriol in comparison with the effect of 1α,25-dihydroxyvitamin D_3, keratinocytes and HaCaT cells were incubated with different amounts of both substances and the concentration-dependent effects on cell proliferation were measured. As shown in Fig. 3A, keratinocytes were more sensitive to incubation with low concentrations ($c \leq 100$ nM) of calcipotriol or 1α,25-dihydroxyvitamin D_3 as compared with HaCaT cells and cell prolif-

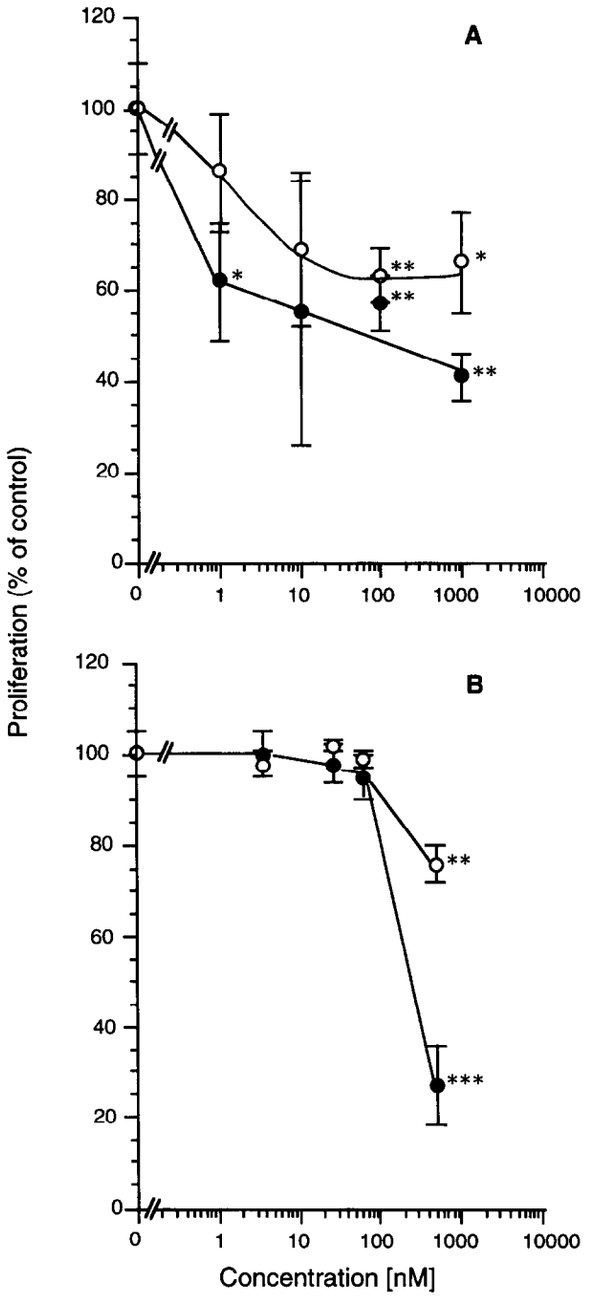

Fig. 3. Antiproliferative effects of 1α,25-dihydroxyvitamin D_3 and calcipotriol on human keratinocytes and HaCaT cells. 5×10^3 keratinocytes/cm^2 (A) and 5×10^3 HaCaT cells/cm^2 (B) were treated with different concentrations of 1α,25-dihydroxyvitamin D_3 (●) or calcipotriol (○). Control media contained 1% ethanol. After 48 h (HaCaT cells) and 72 h (keratinocytes), cell proliferation was determined by the MUH fluorescence assay as described in section 2. Values represent the mean of four experiments and the standard deviation is shown as a vertical bar. *Significantly different from controls at $P < 0.02$. **Significantly different from controls at $P < 0.01$. ***Significantly different from controls at $P < 0.001$.

eration was inhibited by about 40% in the presence of 100 nM calcipotriol or 1α,25-dihydroxyvitamin D_3. However, significant antiproliferative effects of both compounds were also observed in HaCaT cells at higher concentrations (Fig. 3B).

There have been a lot of implications in the literature that the SM cycle plays an important role in the negative regulation of cell proliferation and it has been shown that inducers of SM hydrolysis, such as vitamin D_3 and TNFα, caused growth inhibition of HL-60 cells [28] and human keratinocytes [29]. Furthermore, 4 days after addition of sphingomyelinase to the culture medium of HL-60 cells a decrease in cell proliferation was observed [1] suggesting that SM breakdown and increased levels of ceramides are responsible for the antiproliferative effect. Hence, we used the cell-permeable ceramide, acetylsphingosine (C_2-ceramide), and natural ceramide in our experimental system. C_2-ceramide inhibited cell proliferation of keratinocytes (data not shown) and HaCaT cells (Fig. 4) in the low μmolar range thereby confirming the results from another laboratory [30]. By using conditions which allowed ceramide penetration into cells [16] we could demonstrate that natural ceramide derived from sphingomyelin (ceramide III) or cerebrosid (ceramide IV) inhibited proliferation of HaCaT cells by 21% and 40%, respectively (Fig. 4).

In conclusion, we provide evidence that the SM cycle, a signalling pathway originally described in HL-60 cells, is also found in the immortalized human keratinocyte cell line HaCaT and in human keratinocytes. In addition to the known inducers of SM hydrolysis, 1α,25-dihydroxyvitamin D_3 and TNFα, both cell types responded to the antiproliferative, synthetic vitamin D_3 analogue, calcipotriol. Acetylsphingosine, a cell-permeable, short chain ceramide which is similar to the naturally occurring breakdown product of SM hydrolysis, mimicked the effects of calcipotriol and 1α,25-dihydroxyvitamin D_3 on cell proliferation. Therefore, we suggest that the induction of the SM cycle is one possible mechanism underlying the antiproliferative

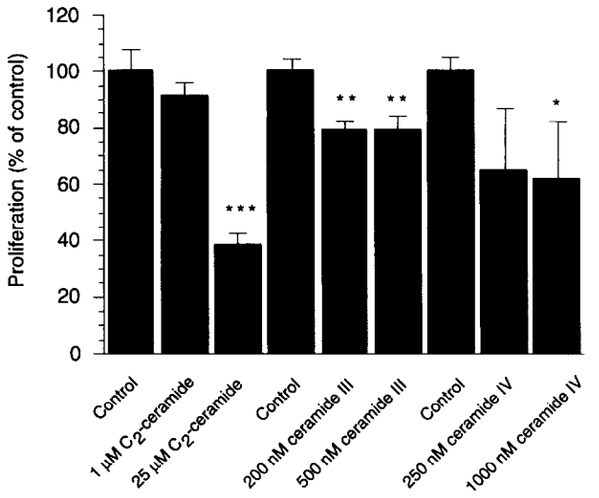

Fig. 4. Antiproliferative effects of C_2-ceramide and natural ceramide on HaCaT cells. 5×10^3 HaCaT cells/cm^2 were treated with different concentrations of C_2-ceramide or with different concentrations of natural ceramide. Control media contained 0.1% dimethyl sulphoxide or 0.56% ethanol/dodecane (98:2, v/v). After 24 h, cell proliferation was determined as described in section 2. Values represent the mean of four experiments and the standard deviation is shown as a vertical bar. *Significantly different from controls at $P < 0.05$. **Significantly different from controls at $P < 0.01$. ***Significantly different from controls at $P < 0.001$.

effect of the non-calcemic vitamin D_3 analogue, calcipotriol, which is used in the topical treatment of psoriasis.

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